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## Molecular mechanism and function of CD40/CD40L engagement in the immune system

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### Summary

During the generation of a successful adaptive immune response, multiple molecular signals are required. A primary signal is the binding of cognate antigen to an antigen receptor expressed by T and B lymphocytes. Multiple secondary signals involve the engagement of costimulatory molecules expressed by T and B lymphocytes with their respective ligands. Because of its essential role in immunity, one of the best characterized of the costimulatory molecules is the receptor CD40. This receptor, a member of the tumor necrosis factor receptor family, is expressed by B cells, professional antigen-presenting cells, as well as non-immune cells and tumors. CD40 binds its ligand CD40L, which is transiently expressed on T cells and other non-immune cells under inflammatory conditions. A wide spectrum of molecular and cellular processes is regulated by CD40 engagement including the initiation and progression of cellular and humoral adaptive immunity. In this review, we describe the downstream signaling pathways initiated by CD40 and overview how CD40 engagement or antagonism modulates humoral and cellular immunity. Lastly, we discuss the role of CD40 as a target in harnessing anti-tumor immunity. This review underscores the essential role CD40 plays in adaptive immunity.

### Keywords

CD40; CD40L; tumor necrosis family; TRAF proteins; humoral immunity; cellular immunity; graft tolerance; tumor immunity

### Introduction

In the initiation of an adaptive immune response, multiple signals are necessary. A primary signal is the engagement of the T-cell antigen receptor (TCR), with polypeptides derived from a protein presented in the context of major histocompatibility complex II (MHC II) on the surface of antigen-presenting cells (APCs) and the binding of native antigen to a cognate B-cell receptor (BCR) complex expressed by B cells. Subsequent secondary signals involve the engagement of costimulatory molecules expressed as receptor and ligand pairs between T cells and APCs and between B cells and T cells, with CD40 ligand (CD40L) expressed by activated T cells engaging CD40 expressed by B cells and APCs (1). Other accessory signals are necessary: the secretion of cytokines functioning to further enhance, modify, and skew the responding effector cells. Although T-cell priming and B-cell activation can occur in

absence of CD40 signals, many cellular and immune functions are defective in the absence of this interaction, underscoring the importance of this ligand/receptor pair in the development of adaptive immunity (2).

Costimulatory molecules are broadly divided into two groups based on their respective homologies to founding members. The CD28/B7 family includes CD28 and cytotoxic T-lymphocyte antigen-4 (CTLA-4), and binds to its ligands CD80 and CD86 (3). The tumor necrosis factor (TNF) receptor (TNFR) family, of which CD40 is a member, also includes TNFR superfamily member 4 (OX40); TNFR superfamily member 13c (BAFF-R); TNFR superfamily member 13b; (TACI), TNFR superfamily member 17 (BCMA); and TNFR superfamily member 11a (RANK) (4). Costimulatory molecules can be further subdivided based on function. For example, molecules containing positive costimulatory function can be grouped together, such as CD40L to CD40 binding, or negative costimulatory function, i.e. CTLA-4 binding CD80/CD86 (5).

The costimulatory receptor CD40 is a 48-kDa type I transmembrane protein and contains a 193 amino acid (aa) extracellular domain, 21 aa leader sequence, 22 aa transmembrane domain, and a 62 aa intracellular domain in human (90 aa in mouse) (6). In the extracellular domain of CD40, there are 22 cysteine residues that are conserved between the members of the TNFR superfamily (6). In regard to expression pattern, CD40 was initially characterized on B cells and is also expressed on dendritic cells (DCs), monocytes, platelets, and macrophages as well as by non-hematopoietic cells such as myofibroblasts, fibroblasts, epithelial, and endothelial cells (7–9).

The ligand of CD40, known as CD154 or CD40L, is a type II transmembrane protein, with a variable molecular weight between 32 and 39 kDa because of post-translation modifications (6). A soluble form of CD40L has been reported that expresses activities similar to the transmembrane form (10, 11). CD40L is a member of the TNF superfamily and is characterized by a sandwich extracellular structure that is composed of a  $\beta$ -sheet,  $\alpha$ -helix loop, and a  $\beta$ -sheet (12). This structure allows for the trimerization of CD40L, which is also a feature of the TNF family of ligands (12). CD40L is expressed primarily by activated T cells, as well as activated B cells and platelets; and under inflammatory conditions is also induced on monocytic cells, natural killer cells, mast cells, and basophils (13). The wide expression of this costimulatory pair indicates the pivotal roles they play in different cellular immune processes.

CD40L/CD40 interactions exert profound effects on DCs, B cells, and endothelial cells, among many cells of the hematopoietic and non-hematopoietic compartments. It has been demonstrated that CD40 engagement on the surface of DCs promotes their cytokine production, the induction of costimulatory molecules on their surface, and facilitates the cross-presentation of antigen (2). Overall, the impact of CD40 signaling ‘licenses’ DCs to mature and achieve all of the necessary characteristics to effectively trigger T-cell activation and differentiation. CD40 signaling of B cells promotes germinal center (GC) formation, immunoglobulin (Ig) isotype switching, somatic hypermutation (SHM) of the Ig to enhance affinity for antigen, and finally the formation of long-lived plasma cells and memory B cells (14). Moreover, it has been shown that the CD40 pathway is essential for the survival of many cell types including GC B cells, DCs, and endothelial cells under normal and inflammatory conditions (15). In a pathogenic setting, the deregulation of CD40 signaling has been observed in multiple autoimmunity diseases. Together, this breadth of functions for CD40 underline the importance this receptor contains during the generation of an acquired immune response (2).

In this review, we describe CD40 signaling and the pathways implicated in this process and discuss work detailing the role of CD40 signaling in both humoral and cellular immune responses. Lastly, we will describe how CD40 targeting has led to novel anti-tumor therapies.

## TRAF-dependent and -independent CD40 signaling

The engagement of CD40 by CD40L promotes the clustering of CD40 and induces the recruitment of adapter proteins known TNFR-associated factors (TRAFs) to the cytoplasmic domain of CD40 (15). The TRAF proteins activate different signaling pathways including the canonical and non-canonical nuclear factor  $\kappa$ B (NF  $\kappa$ B)-signaling pathways, the mitogen-activated protein kinases (MAPKs), phosphoinositide 3-kinase (PI3K), as well as the phospholipase C (PLC  $\gamma$ ) pathway (15). Recent evidence indicates that signaling may occur independent of the TRAF proteins, as well as with Janus family kinase 3 (Jak3), which was found to be able to bind directly to the cytoplasmic domain of CD40. Binding of Jak3 has been shown to induce the phosphorylation of signal transducer and activator of transcription 5 (STAT5) (16, 17). Together, these complex pathways elicit the essential signals mediated through CD40 to impart its diverse cellular processes (Fig. 1).

Perhaps the best-characterized signaling pathways initiated during CD40 activation are the canonical and non-canonical NF  $\kappa$ B pathways, which lie downstream of the recruitment of the TRAF proteins to the CD40 cytoplasmic domains. The canonical NF  $\kappa$ B pathway begins with the formation of the inhibitor of  $\kappa$ B (I  $\kappa$ B) kinase (IKK) complex, containing the catalytic subunits IKK  $\alpha$ , IKK  $\beta$ , and the regulatory subunits IKK  $\gamma$  or NEMO. The activation of NF  $\kappa$ B leads to the ubiquitin and proteasomal-dependent degradation of the I  $\kappa$ B complex, inducing the translocation of NF  $\kappa$ B heterodimers p50/p65 and p50/c-Rel to the nucleus. The non-canonical NF  $\kappa$ B pathway is induced when the protein kinase NF  $\kappa$ B-inducing kinase (NIK) activates IKK  $\alpha$ , leading to a limited proteolysis of the precursor p100 that produces p52 protein, which associates with avian rel B and translocates into the nucleus. The result is that both signaling pathways can lead and regulate different target genes (18).

The TRAF family contains six members, designated TRAF1 through TRAF6, with all members driving cellular processes upon TNFR family engagement with their respective ligands. Characteristic of all TRAFs, members have a highly conserved carboxyl domain, which is called the TRAF domain. Upon ligand engagement, TRAF proteins are recruited to canonical aa motifs contained in the cytoplasmic domains of the TNFR family, with the TRAF domain engaging its cognate aa motif located in the TNFR cytoplasmic domains. TRAF proteins, with exception of TRAF1, contain an amino-terminal ring finger domain followed by five zinc fingers and a coiled-coil domain, with this structure called the zinc finger domain. The genetic ablation of the zinc finger domain generates a dominant negative TRAF protein that inhibits any induction of signaling mediated by the normal TRAF, demonstrating that this domain is essential for signaling (15).

Upon engagement with CD40L, CD40 directly or indirectly recruits TRAF1, TRAF2, TRAF3, TRAF5, and TRAF6 to its cytoplasmic domains. The canonical consensus binding sites for TRAF1, TRAF2, and TRAF3 are situated at the membrane distal domain of the intracellular tail of CD40 and is demarcated by the aa sequence PxQxT (19, 20). The insertion of mutations at the PxQxT domain disrupts the binding of TRAF2/3 to the CD40 cytoplasmic tail and dampens CD40 signaling by abrogating the NF  $\kappa$ B, MAPK8 (Jnk), and p38 pathways during ligand engagement (21, 22). Our laboratory found a second, non-canonical binding site for TRAF2 at the carboxy-terminus of CD40 tail (23). Through this TRAF2 binding site, NF  $\kappa$ B signaling is capable of being induced to control several B-cell functions (24). TRAF6 is recruited to the membrane proximal domain of CD40 to the

consensus sequence QxPxEx (15). Through recruitment of TRAF proteins and their binding to different sites of CD40, TRAFs are essential in driving CD40-mediated signaling.

By elucidating the crystal structure of a portion of the CD40 cytoplasmic tail bound to TRAF2, McWhirter *et al.* (25) revealed the complexity behind the conformational changes driven by CD40L engagement by revealing the oligomerization that occurs with CD40 in complex with TRAF2. Upon engagement with CD40L, a conformational change occurs in CD40 that exposes the docking site for TRAF2 at the canonical motif, and thus a trimeric TRAF2 is able to interact with three cytoplasmic tails of CD40 (25). In order to drive fulminant CD40 signaling, higher levels of oligomerization are required than trimerization, and it is likely that these different orders of oligomerization may result in differential recruitment of adapter and kinase proteins, particularly those that bind with low affinity to the cytoplasmic domains of CD40 such as TRAF1 or TRAF6 (26, 27). These differences in oligomerization likely account for the breadth of biologic activities induced by different monoclonal antibodies to CD40 (28).

TRAF1 protein expression is heightened during CD40 (29, 30). There is evidence demonstrating that TRAF1, as a result of the absence of a zinc finger domain, plays an important role in regulating the signaling of the other TRAF proteins (15). The TRAF1 binding site motif contained by CD40 overlaps with the binding sites for TRAF2 and TRAF3, and it appears that TRAF1 can bind weakly to the CD40 cytoplasmic domains in the absence of TRAF2 (20). The physiological implications of this observation are unclear. In APCs deficient in TRAF1, a reduction is observed in the recruitment of TRAF2 to the cytoplasmic domain of CD40, with a corresponding increase of TRAF2 degradation (31). These results were confirmed by Xie *et al.* (32) when it was demonstrated that CD40 stimulation of a B-cell line deficient in TRAF1 increases the amount of TRAF2 recruited to lipid rafts and increases the degradation of TRAF2 and TRAF3. In addition, data presented demonstrate that the recruitment of both TRAF1 and TRAF2 to CD40, during CD40L engagement, is required for activation of the canonical NF- $\kappa$ B pathway. This was shown using B-cell line deficient for TRAF2 and TRAF1, which exhibit attenuated activation of canonical NF- $\kappa$ B compared with single TRAF-knockout B cells (32). Together, these results suggest that TRAF1 can interact and regulate the recruitment and degradation of TRAF2. Moreover, both TRAF proteins can cooperate in the activation of the canonical NF- $\kappa$ B pathway.

During CD40 engagement, the recruitment of TRAF2 to the tail of CD40 leads to the activation of the Jnk, p38, and thymoma viral proto-oncogene 1 (Akt) pathways. This was shown using TRAF2-deficient mouse embryonic fibroblasts (MEFs) and B cells, which exhibited attenuated activation of these pathways upon CD40 engagement (33–35). Similar results were observed in B cells transfected with a dominant negative TRAF2 (34). The engagement of CD40 induces the recruitment of TRAF2 with the kinase mitogen-activated protein kinase kinase kinase 1 (MEKK1) to CD40 tail. In experiments where CD40 was engaged on B cells lacking MEKK1, the activation of Jnk and p38 was blunted (36). MEKK1 drives the phosphorylation of Jnk and p38 and thus activates these respective pathways (36).

Despite its activating role in the aforementioned pathways, TRAF2 appears to be a negative regulator of the non-canonical NF- $\kappa$ B pathway. The ablation of TRAF2 induces the accumulation of NIK in MEFs (37). Recent studies have elegantly demonstrated that TRAF2 cooperates with TRAF3 to constitutively degrade NIK, with engagement of another TNFR, BAFF-R, releasing this degradation and allowing for activation of the non-canonical NF- $\kappa$ B pathway (38). It is clear that engagement of CD40 activates this pathway in a similar mechanism. Without CD40 stimulation, a complex is formed between family members of

the cellular inhibitor of apoptosis (cIAP) 1 and 2, which interact with TRAF2, and which in turn interacts with TRAF3 and NIK (Fig. 2). Under these conditions, cIAP1/2 induces the degradation of NIK and antagonizes the non-canonical NF- $\kappa$ B pathway (39, 40). In contrast, after CD40/CD40L engagement, the complex is destabilized, leading the recruitment of TRAF2 and TRAF3 to the cytoplasmic domains of CD40, with activation of the non-canonical NF- $\kappa$ B pathway resulting from the subsequent accumulation of NIK as a result of TRAF2 and TRAF3 degradation following recruitment to CD40 (15). The degradation of TRAF2 is the result of self-degradation, because of its E3-ubiquitin ligase property, whereas TRAF3 degradation is dependent of cIAP1/cIAP2 (40, 41). Together, these results show that cIAP1/2 has a dual role in CD40 signaling, that in a steady state, cIAP1/2 inhibits the non-canonical NF- $\kappa$ B signaling by degradation of NIK, whereas upon CD40 activation, cIAP1/2 promotes TRAF3 degradation, which releases NIK and promotes activation of the alternative NF- $\kappa$ B pathway.

Initial descriptions of the role TRAF3 plays in CD40 signaling proposed that it functioned as a negative regulator of the canonical NF- $\kappa$ B pathway (42). This hypothesis was based on experiments where a TRAF3-dominant negative protein expressed in B cells leads induction of the canonical NF- $\kappa$ B and Jnk signaling pathways upon CD40 engagement (42, 43). These results were corroborated in experiments where CD40 signaling was studied in TRAF3-deficient B cells; it was also observed that Jnk phosphorylation and an accumulation of NIK was enhanced, as well as the recruitment of TRAF2 to lipid rafts suggesting that TRAF3 regulates TRAF2 and NIK, as was discussed above (39, 40, 43). In contrast, it was observed in epithelial cells that the enforced overexpression of TRAF3 induces canonical NF- $\kappa$ B signaling during CD40 stimulation, suggesting that TRAF3 has different roles in different types of cells (44, 45). Therefore, in B cells, TRAF3 negatively regulates canonical and non-canonical NF- $\kappa$ B pathways, as well as Jnk signaling; whereas in epithelial cells, it is able to induce canonical NF- $\kappa$ B signaling. Additional experiments are necessary to determine the role of TRAF3 in non-canonical NF- $\kappa$ B and Jnk signaling in epithelial cells.

Very little is known about the role of TRAF5 in CD40 signaling although TRAF5 is incapable to bind directly to CD40 cytoplasmic domains. During CD40 signaling, TRAF5 forms heterotrimers with TRAF3 (15). The role of TRAF5 signaling, during CD40 stimulation, was shown in B cells treated with small interfering RNAs (siRNAs) specific for TRAF5 or deficient for TRAF5 where canonical and non-canonical NF- $\kappa$ B pathways were ablated. That is translated in a reduction of the antibody production, proliferation, costimulatory molecules expression (46, 47). Further experiments are necessary to fully understand TRAF5 function in CD40 signaling.

Like other TRAF proteins, the physical interaction of TRAF6 with CD40 was demonstrated by the yeast two-hybrid assay (15). In TRAF6-deficient MEFs or epithelial cells where TRAF6 has been silenced by siRNA, these cells exhibited a reduction or abrogation in the activation of canonical NF- $\kappa$ B, Jnk, p38, and Akt, upon CD40 engagement. Interestingly, TRAF6 is able to interact with TRAF2 and thus regulate in a positive way CD40 signaling (21). Recently, Rowland *et al.* (48) have shown that in B cells expressing CD40 void of a TRAF6 binding site, TRAF6 was still capable of interacting with TRAF2. This suggests that TRAF6 may still have a functional role in CD40 signaling, without binding directly to CD40. Under these conditions, CD40 stimulation was capable of enhancing CD80 upregulation and inducing Jnk activation in comparison to B cells deficient in TRAF6. These data implicate TRAF6 as regulating some of its physiological functions through its interactions with TRAF2 and independent of its recruitment to the TRAF6 binding site of CD40 (48).



CD40 can induce the recruitment of TRAF6/Casitas B-lineage lymphoma b (Cbl-b)/Casitas B-lineage lymphoma (c-Cbl)/PI3K complex, which leads to Akt phosphorylation. The members of Cbl family are adapter molecules, which have E3-ubiquitinating ligase properties (49). Depending on the cell type, they regulate positively or negatively a wide range of kinase and adapter proteins such as PI3K, Rous sarcoma oncogene (Src) tyrosine kinases, spleen tyrosine kinase, growth factor receptor bound protein 2, and Src homology 2 domain-containing transforming protein C1 (49). In studies using Cbl-b-deficient DCs stimulated *in vitro* with CD40L, an ablation was observed in Akt activation without effect in the induction of the canonical NF- $\kappa$ B pathway. These findings suggest that in DCs, the Akt activation is independent of NF- $\kappa$ B signaling (49). As mentioned above, Cbl-b induces Akt phosphorylation through PI3K, and after the CD40 engagement, the blocking of PI3K abrogates Akt activation, demonstrating that in DCs, the Cbl-b activates PI3K, which induces the phosphorylation of Akt during CD40 signaling (50).

Physiologically, the blocking of PI3K drastically affects the survival of DCs (51). The mechanism that involves the anti-apoptotic effect of PI3K/Akt activation is dependent on the inhibition of the pro-apoptotic proteins, caspase 9, B-cell leukemia (Bcl)/lymphoma 2-associated agonist of cell death (50, 52, 53). Recently, a second mechanism was determined; PI3K can activate the mammalian target of rapamycin, which induces the expression of the anti-apoptotic protein caspase 8 and Fas-associated via death domain-like apoptosis regulator p43 (cFLIPp43) (50). Therefore, CD40, probably through TRAF6, induces the activation of PI3K signaling that protects the cell from apoptosis.

In contrast to the result described above, B cells deficient in Cbl-b have an increase of NF- $\kappa$ B and Jnk pathways, suggesting that in B cells Cbl-b is a negative regulator of these signaling pathways (54). In this case, Cbl-b is recruited through TRAF2 to cytoplasmic tail of CD40 and the lack of Cbl-b increases TRAF2 recruitment to CD40, showing a negative role of Cbl-b in the recruitment of TRAF2 (54). Together, these results propose that in different cell types, Cbl-b can regulate different pathways dependent on which TRAF proteins are interacting.

Most of the studies investigating CD40 signaling demonstrate pathways dependent on TRAF proteins. However, it was observed in B cells, the membrane proximal region of the CD40 tail contains a binding domain for Jak3 (55). Revy *et al.* (56) demonstrated that in B cells, upon CD40 stimulation, Jak3 is not phosphorylated, suggesting that the recruitment of Jak3 to the cytoplasmic membrane of CD40 is not functional. However, monocytes are able to induce Jak3 phosphorylation after CD40 engagement (56). Furthermore, the inhibition of Jak3 in APCs, upon CD40 stimulation, blocks the maturation of APCs (16, 17). Additionally, Jak3 induces the transcription factor STAT5, which can dimerize and translocate to the nucleus, and leads the gene expression of the inflammatory cytokines, such as TNF- $\alpha$ , interferon- $\gamma$  (IFN- $\gamma$ ), and interleukin-6 (IL-6) (57). These results suggest that Jak3 is able to bind to CD40 and induce the maturation of DCs through STAT5.

In conclusion, CD40 principally mediates signaling through TRAF proteins, which can activate or inhibit different signaling pathways, dependent upon the cell type. TRAF proteins drive a wide range of cellular and immune processes and understanding these pathways will improve our ability to develop strategies to therapeutically intervene in a vast array of immune related diseases.

## CD40 and humoral immunity

During initiation of a thymus-dependent (TD) humoral immune response, CD40 signaling by B cells is required for the generation of high titers of isotype-switched, high affinity antibody and for the development of humoral immune memory. The engagement of CD40

expressed on the surface of antigen-activated B cells by CD40L expressed on activated CD4<sup>+</sup> T cells is essential for the initiation and progression of a TD humoral immune response. CD40 engagement triggers B-cell intercellular adhesion, sustained proliferation, expansion, differentiation, and antibody isotype switching *in vitro* (58–61). *In vivo*, CD40 engagement is required for GC formation and progression, as well as antibody isotype switching and affinity maturation, with these processes essential for the generation of memory B cells and long-lived plasma cells (62–64). The blockade of CD40/CD40L interactions through the use of blocking antibodies, genetic ablation of CD40L, or genetic ablation of CD40 completely abolishes TD humoral immunity (58).

### Identification of CD40/CD40L as essential for TD humoral immunity

Historically, studies in regulation TD humoral immunity have detailed an essential role for the physical collaboration between T cells and B cells for an effective TD humoral immune response to be generated (65). This cellular collaboration is manifest as a physical interaction between antigen-activated B cells presenting antigen-derived peptides in the context of MHC class II to cognate CD4<sup>+</sup> T-helper cells (Th cells) specific for the class II-restricted peptides being presented (66, 67). As a result of this immunological synapse, the Th cell provides the B cell with contact-dependent and -independent stimuli. Initially, it was thought that local release of cytokines was responsible for the ability of T cells to induce B-cell growth and differentiation. However, cytokines such as IL-4, IL-5, IL-2, IL-10, and IFN- $\gamma$  alone or in combination were unable to reconstitute the abilities of T cells to stimulate sustained B-cell growth and differentiation (58, 68–71). Based on these findings, it was predicted that further cell-contact mediated mechanisms of T/B cell crosstalk existed (72). This prediction was supported by the observation that plasma membrane fractions from activated Th cells were capable of driving B cells into cell cycle, with this induction occurring independent of cytokines (73–75). The molecule through which B cells receive this contact-dependent stimulus from T cells was identified as CD40 through experiments where agonistic monoclonal antibodies raised against CD40 were found to drive B cells into cell cycle and by using a CD40-Ig fusion protein to antagonize the ability of plasma membrane fractions to activate B cells *in vitro* (60, 76). By using the CD40-Ig fusion protein, CD40L expressed by activated Th cells was identified, isolated, and cloned (76, 77). Furthermore, a monoclonal antibody was raised against CD40L that antagonized the interactions of CD40 with CD40L both *in vitro* and *in vivo* (64, 76). This antibody (clone MR1) has proven to be invaluable in studies determining the role of CD40/CD40L in the regulation of humoral immunity.

### The *in vivo* role of CD40/CD40L interactions during a humoral immune response

During infection with a pathogen, either whole pathogen or its particulates enter the lymph and the proximal draining lymphoid organs or, in the case of systemic infection, enter the spleen through the blood. Direct encounter of cognate antigen by naive follicular (NF) B cells residing in the B-cell follicles can occur. Alternatively, antigen can be acquired by tissue-resident DCs in the periphery that then home to the secondary lymphoid organs and deliver native antigen to cognate NF B cells (78–80). Lymphoid-homing DCs also present processed antigen in the context of MHC class II to Th cells in the T-cell zones of the secondary lymphoid organs, leading to the clonal activation of antigen-specific T cells. CD40/CD40L interactions between antigen-presenting DCs and Th cells are essential for the maturation and survival of DCs. In turn, the CD40-dependent maturation of DCs leads to the sustained expansion and differentiation of antigen-specific T cells.

The response of NF B cells to TD antigen and T-cell help has been well defined as a sequence of events that are temporally ordered. B-cell activation as defined by the induction of a litany of cell surface molecules can be readily observed hours after TD activation.



Shortly thereafter, entry into the cell cycle can be documented. These early proliferating cells differentiate into plasmablasts secreting germline-encoded IgM and then IgG, with these plasmablasts located outside of the B-cell follicles (days 2–12 postimmunization). Activated B cells also seed secondary follicles and rapidly proliferate and interact with Th cells in a GC reaction, where isotype switching and SHM of the BCR occurs (days 9–20). GC B cells further differentiate into long-lived plasma cells and memory B cells containing high-affinity BCRs of the switched isotypes (days 20+) (81–83).

It is clear that CD40L plays a critical role in controlling B-cell fate during many of the aforementioned checkpoints in B-cell growth and differentiation. When Th cells are activated by DCs within the T-cell zone during a primary TD response, they transiently express CD40L (84). These activated Th cells then home toward the B-cell follicles and position themselves at the border of the B-cell follicles and T-cell zone where they encounter activated and cognate B cells, with CD40/CD40L interactions occurring between the two cell types (85). Th cells expressing CD40L are detectable at high frequencies by days 2–5 after immunization and co-localize with plasmablasts secreting antibody specific for the immunizing antigen in the T-cell zone (84). Th cells expressing CD40L are essential in activating NF B cells to differentiate into plasmablasts as blockade of CD40/CD40L interactions between responding Th cells and cognate B cells completely ablates the plasmablast response (64). In contrast to the TD immune response, abrogation of the CD40/CD40L pathway has no impact on the thymus-independent (TI) antibody response. When mice are immunized against the TI-type II antigen TNP-Ficoll, no difference in the strength of the humoral immune response is seen between mice treated either with anti-CD40L antibody or with control antibody (64). Similar results were observed in studies comparing the ability of CD40L knockout mice to mount an immune response to TNP-Ficoll in comparison to wildtype mice (86). In summation, CD40 signaling in antigen-responding B cells is essential for their differentiation into plasmablasts in response to a TD antigen.

### **CD40/CD40L interactions in the initiation and progression of the GC**

In parallel to the plasmablast response, a subset of oligoclonal antigen-responding NF B cells which have been similarly activated in the T-cell zones colonize the B-cell follicles where they form GC structures (81, 83). The fate of whether a responding B cell differentiates into a plasmablast or whether it seeds a GC is dictated by a number of B-cell intrinsic or extrinsic factors. The intrinsic factors include the germline-encoded affinity of the B cell for antigen, with this affinity exerting a tremendous impact on B-cell fate. That is, high initial affinity BCR tends to drive the B cells into the plasma-blast lineage, whereas moderate affinity imprints the B cells to seed the GC (87–90). Extensive triggering via CD40/CD40L is an example of a cell extrinsic factor that modifies B-cell fate. There clearly is a range of CD40 signaling that is ‘acceptable’ in inducing a GC response. In the absence of CD40 stimulation, no GC response is observed. As CD40L signaling is provided, a GC response initiates and matures. However, in the presence of enhanced CD40 stimuli during a primary immune response, either with agonistic anti-CD40 antibodies or elevated T-cell help, B cells are selectively triggered to differentiate down the plasmablast lineage and divert from a GC response (91). In this situation of agonistic anti-CD40, the antigen-responding B cells uniformly differentiate into plasmablasts localized outside of the B-cell follicles, and no GC is formed. A similar scenario, albeit to a less severe degree, occurs when the numbers of activated, cognate T cells are enhanced by an order of magnitude. After priming with the carrier protein keyhole limpet hemocyanin (KLH) 7 days prior to immunization with the hapten/carrier (4-hydroxy-3-nitrophenyl) acetyl conjugated to KLH to simulate enhanced T-cell help, responding B cells are again shuttled from seeding a GC and instead differentiate into plasmablasts; although in this case GCs do appear (91). It is

clear that the strength and duration of a CD40 signal, while required for the onset of humoral immunity, also has a substantial impact on the fate of antigen-responding B cells (91).

Once committed to the GC lineage, B cells encounter a specialized subset of activated Th cells called T follicular helper cells (T<sub>FH</sub> cells), that provide the necessary cytokine and cell-contact signals, including CD40L, required to sustain the GC reaction (92–94). By means yet unclear, after T/B-cell encounter at the edges of the periarteriolar lymphoid sheath and B-cell follicles, a subset of activated Th cells further differentiate into T<sub>FH</sub> cells and are licensed to enter the B-cell follicles by the heightened expression of the chemokine receptor chemokine (C-X-C motif) receptor 5 (CXCR5) (93, 95). The chemokine ligand for CXCR5 is chemokine (C-X-C motif) ligand 13 (CXCL13), and this chemokine coordinates the positioning and structure of the B-cell follicles as mature B cells, which express CXCR5 (96–100). T<sub>FH</sub> cells are characterized by their expression of CD40L, ICOS, CXCR5 and the secretion of the cytokine IL-21, which stimulates B-cell proliferation, isotype switching, and differentiation into plasma cells (93). CD40L has been shown to be critical for T<sub>FH</sub> cells function.

As the GC reaction matures, the structure becomes polarized into light and dark zones, with each zone situated to play a role in GC B-cell competition for antigen and T-cell help (83). The GC dark zone is so named because of the presence of a high density of rapidly dividing B cells, called GC centroblasts, whose large nuclei project a dark appearance during histological analysis. It is at the GC dark zone that SHM occurs (101). The GC dark zone is maintained by the chemokine, chemokine (C-X-C motif) ligand 12 (CXCL12), with this chemokine secreted by the resident stromal cells (102). Accordingly, the receptor for this chemokine, chemokine (C-X-C motif) receptor 4 (CXCR4), is expressed on GC centroblasts (102, 103). The GC light zone is situated at the pole of antigen entry, and within this zone resides follicular dendritic cells (FDCs) on which antigen complexes are tethered by binding complement receptors CD21 and CD35 and the Fc  $\gamma$ RIIb (104). At the GC light zone, FDCs and light zone stromal cells secrete CXCL13 and it is here that T<sub>FH</sub> cells are found (102, 103). GC B cells residing in the light zone are called centrocytes and are characterized by the expression of CXCR5 (102). Within the GC light zone, the interactions between centrocytes, T<sub>FH</sub> cells, and complexed antigen occur in a critical competition and result in the selection of centrocytes exhibiting the highest affinities for antigen, as they receive survival and differentiation signals through their BCR and through CD40 engagement. It should be noted that the role of immune complexes on FDC in affinity maturation has been brought into question by Shlomchik and coworkers (105).

GC B cells expressing BCRs exhibiting inferior affinities for antigen are deleted by Fas-dependent apoptosis as they do not receive sufficient BCR and CD40 survival signals. The engagement of CD40 by a GC centrocyte during a productive immune synapse with a T<sub>FH</sub> cell induces cellular survival. This was initially shown in studies observing that GC B cells could be rescued *in vitro* with the administration of agonistic anti-CD40 antibody or CD40L (106, 107). The engagement of CD40 by centrocytes protects the cell from undergoing Fas-mediated apoptosis by inducing the anti-apoptotic proteins Bcl/B-cell lymphoma  $\times$  (Bcl-XL) and c-FLIP (108–110). It is clear that T<sub>FH</sub> cells provide centrocytes with signals in addition to CD40L in order to propagate survival and differentiation to memory and plasma cells, with IL-21 occupying such a role in PC differentiation (93). Classic *in vitro* studies by MacLennan and coworkers (106) observed that centrocytes are induced to differentiate into memory B cells upon culture with activated memory Th cells but not activated naive Th cells, indicating that further signals may exist (106). How these other signals, which are derived from T<sub>FH</sub> impact centrocyte differentiation into memory B cells and PCs are in the process of being elucidated.

CD40 signaling from activated T cells at the inductive phase of the GC response as well as from T<sub>FH</sub> cells throughout the duration of the GC response is essential for the completion of the GC response. Blockade of CD40/CD40L interactions or genetic ablation of the CD40L or CD40 gene prevents the clonal proliferation of antigen-responding B cells as well as blocks the generation of the GC structure by preventing these cells from receiving signals from activated Th cells through CD40 (63, 64, 86). As such, if anti-CD40L antibody is injected at the peak of the GC response, the GC is immediately shut down (111). After anti-CD40L antibody administration, the Ig affinity of the plasma cells in the bone marrow was decreased in comparison to control antibody-treated mice, supporting the hypotheses that (i) affinity maturation occurs in the GC and progresses over time, and (ii) CD40/CD40L interactions are essential for this progression. Together, these data indicate that CD40 signaling initiates and propagates the GC, and that abrogating this reaction through CD40 block prevents the full benefits of the GC, as measured by affinity maturation.

### The contributions of TRAF recruitment during CD40 signaling on B-cell fate

Early events in the CD40 signaling cascade control the fate of antigen-specific B cells. Upon CD40 multimerization by CD40L, TRAFs are recruited to the CD40 cytoplasmic tail as discussed in detail earlier in this review. The respective contributions of TRAFs 1, 2, 3, 5, and 6 recruitment to CD40 in regulating humoral immunity was studied *in vivo* by generating transgenic mice containing CD40 receptors with mutations in defined CD40 binding sites for TRAFs (24, 112–114). Upon CD40L binding to CD40, TRAF2 and TRAF3 directly bind to the cytoplasmic tail of CD40. TRAF1 and TRAF 5 are also recruited to CD40 through indirect associations with CD40-bound TRAFs (20, 115, 116). Through disruption of TRAF6-binding site within the CD40 cytoplasmic domain, we selectively ablated affinity maturation and the generation of plasma cells after immunization. Mutagenesis of both the TRAF6 and TRAF2–TRAF3 sites was essential for arresting GC formation in response to immunization. CD40-induced B-cell proliferation and early Ig production occurred even when all TRAF sites were ablated. These studies demonstrated that specific CD40–TRAF associations control well-defined aspects of humoral immunity (112). The fact that the B-cell proliferative response was intact in mice where both the TRAF2–TRAF3 and TRAF6 sites were ablated suggested another functional site in the CD40 cytoplasmic tail. Indeed, our laboratory identified a second non-canonical TRAF2 binding motif in the C-terminus of CD40 (23, 24). All early B-cell activation events, including plasmablast generation are intact in the transgenic mice that express only the c-terminal TRAF2 binding motif in the CD40 tail. When immunized with T-dependent antigen, the mice are still impaired to develop GC demonstrating that either or both, TRAF3 and TRAF6 are necessary to form GC structures (23, 24). In summary, TRAFs are essential intermediaries in CD40-induced humoral immunity.

Perhaps the most striking example of how essential CD40/CD40L interacts are in generating humoral immunity is the clinical manifestations that arise in humans carrying mutations in the CD40L gene which generate non-functional CD40L. This syndrome is X-linked hyper-IgM syndrome and is characterized by reduced levels of serum IgG, IgA, and IgE and increased levels of IgM (117). Because of inactivation of functional CD40L, TD immune responses in these patients are severely impaired, including the generation of GCs, memory B cells, and isotype-switched plasma cells secreting protective antibody. Patients with CD40L deficiency are thus susceptible to life-threatening bacterial infections. Thus, the importance of CD40/CD40L interactions in controlling humoral immunity has been displayed in an unfortunate clinical presentation.

## The role of CD40/CD40L in cell-mediated immunity

There is little doubt as to the essential role of CD40/CD40L in the development of TD humoral immunity. While initially thought to be only involved in regulating TD immunity, CD40/CD40L is also critical in the development of cell-mediated immunity (CMI). There is clear and unquestionable evidence that the development of some CMI responses require CD40/CD40L (118). This is most evident in the role of this ligand–receptor pair in the development of cytotoxic T cells to tumors, virus, and alloantigens (119, 120) and its role in the development of a litany of T-cell-dependent autoimmune diseases (121). In this review, we discuss its role in regulating allograft tolerance and rejection, as a model that clearly delineates the role of this ligand–receptor pair in controlling critical checkpoints in the development of CMI.

One of the first pathways to be targeted in an attempt to modify allograft rejection was the CD28/B7 pathway. It was demonstrated that by using a CTLA-4-Ig (a soluble form of the high-affinity receptor to B7 molecules), long-term allograft survival of islet cells (122, 123) and cardiac allografts (124) could be achieved. By competitively binding to B7 molecules, CTLA-4-Ig blocked CD28 signaling to the T cells, therefore limiting activation of the alloreactive T-cell compartment. Other means by which to disrupt T-cell activation have been through the blockade of the CD40/CD40L pathway. Originally, it was thought that anti-CD40L antibody monotherapy would be effective because it limited APC maturation and downmodulated the B7–CD28 interaction, resulting in the lack of a signal 2 and T-cell energy (125, 126). However, anti-CD40L antibody as a monotherapy has not been successful in inducing allograft tolerance (127, 128). Studies by Waldmann and coworkers (129) indicate that failure to induce tolerance using antibody anti-CD40L therapy is insufficient because of its inability to block rejection elicited by CD8<sup>+</sup> T cells. Highly immunogenic allografts, such as those of heart and skin, require stronger tolerogenic therapies. Among these, the combination of costimulatory blockade with immunosuppressive drugs (rapamycin with antibody anti-CD40L and CTLA-4-Ig) has been shown to result in long-term graft acceptance (130). Interestingly, the effect of cyclosporine A impedes the tolerogenic effects of anti-CD40L antibody and CTLA-4-Ig. The explanation for this resides in the ability of cyclosporine to block IL-2 production and likely the expansion of regulatory T cells (131).

Given the failures of anti-CD40L antibody monotherapy in many graft systems, we asked if we could pretolerize a host to allow that host to accept an allograft. The specific system that we and others (132–137) have exploited to understand the role of CD40L in the regulation of graft rejection involves the pretolerization of the host with alloantigen (donor-specific transfusion, DST) and anti-CD40L antibody, followed by grafting. In this system, donor alloantigen is provided by an intravenous infusion of T-depleted donor splenocytes or heparinized whole blood. When used in combination with anti-CD40L antibody, this has been shown to induce allospecific T cell tolerance, as measured by suppression of both CD8<sup>+</sup> and CD4<sup>+</sup> alloreactive T cells (127–129, 138) and enhanced persistence of allografts of skin, kidney, pancreatic islets, heart, and lung (132–137).

The underlying cellular and molecular mechanisms that account for the DST and anti-CD40L antibody T-cell tolerance have been studied (135). Studies into this phenomenon have provided insights into the process of allospecific tolerance, peripheral tolerance, and the role of regulatory T cells in the survival of allografts. Blockade of CD40L by anti-CD40L antibody impairs the maturation of the host DCs (upregulation of CD80/86) and incapacitates the abilities of these DCs from inducing productive T-cell activation. As such, the alloantigen-presenting, non-matured DCs induce abortive activation and anergy of the host alloreactive T-cell pool. Among the most relevant findings were that indirect

presentation of alloantigen was essential for tolerance induction. Thus, the infused DST was rapidly processed by host APCs and presented via indirect presentation to the host alloreactive T cells in a tolerogenic manner because of the CD40L blockade. The tolerogenic impact of DST and anti-CD40L antibody in this system was to induce a rapid (days 3–4), systemic, abortive expansion of the alloreactive T cells that resulted in profound anergy. One additional unique feature of this system was that we could quantify the magnitude of the T-cell unresponsiveness by purifying anergic cells. In these cases, the unresponsiveness was determined to be >95% on a per-cell basis. Hence, infusion of DST and anti-CD40L antibody resulted in a pre-emptive induction of tolerance within the alloreactive T-cell compartment, thereby silencing the alloreactive response days prior to the time when the allograft had the opportunity to elicit an immune response (135).

We now envision that the infused DST rapidly undergoes apoptosis and is presented by host APCs. It is of interest to note that anti-CD40L antibody may facilitate the apoptosis of the DST by depriving it of a CD40 signal. At the same time, this blocking antibody impairs the maturation of host APCs, committing them to the tolerogenic presentation of DST-derived allopeptides. Delivery of peptides via apoptotic cells appears to be an extremely efficient means to induce peripheral tolerance. In analogous studies, Steinman and coworkers have shown that B cells deficient in transporter associated with antigen processing 1 protein and hyperosmotically loaded with OVA can induce abortive expansion and anergy of OVA-specific cytolytic T lymphocytes (CTLs) *in vivo* via indirect presentation (139). Similarly, antigens expressed on dying pancreatic cells (140) induce tolerance via indirect presentation. More recently, antigens targeted directly to defined DC surface molecules (such as DEC-205) and antigens delivered to immature DCs have been shown to induce profound antigen-specific tolerance, as predicted by these earlier studies.

CD40-dependent maturation of DCs is far more complex than simply the upregulation of costimulatory molecules. As a consequence of CD40 signaling of DCs, there is heightened expression and increased stability of the MHC/alloantigen complex (141, 142). Furthermore, increased life span of the DC is considered a major factor in the success of CD40-triggered DCs in driving CMI. CD40-mediated survival is based on the upregulation of Bcl-XL, an anti-apoptotic factor and is NF- $\kappa$ B dependent (143, 144). Passive apoptosis of T cells induced by the absence of growth/survival factors, such as IL-2, IL-7, and IL-15 imposed by CD40L blockade also can impact on T cell immunity (145–147).

Central to the induction of alloreactive T-cell tolerance by many interventions involving costimulatory blockade is the induction of regulatory T cells (148). The functional importance of this population has been repeatedly demonstrated by graft loss resulting from anti-CD25 antibody treatment and the observations of overt infiltration of regulatory T cells into the graft observed by immunohistochemical analysis (135, 149). By using forkhead box p3 (Foxp3)-expressing T cells (150) or Foxp3-green fluorescent protein positive T cells (Victor C. de Vries, Randolph J. Noelle, unpublished observation), it was confirmed that the tolerance can be transferred by just the regulatory T cells. However, one cannot discount the massive reduction of alloreactive effector T cells by anti-CD40L antibody and DST in facilitating graft survival. As anticipated, it has been shown that the maturation status of DCs regulates the generation of antigen-specific regulatory T cells. Likewise, strong costimulation by DCs after treatment with agonistic CD40 and/or lipopolysaccharide diminished regulatory T cells expansion and, CD40-deficient DCs or blocking with anti-CD40L antibody greatly enhanced regulatory T cells development from naive TCR-transgenic T cells (151, 152) to regulatory T cells.



### Combination therapies using CD40L blockade

From studies using anti-CD40L antibody, it is clear that although graft survival is significantly prolonged, indefinite tolerance is not achieved. In murine models, permanent allograft survival using anti-CD40L antibody and DST requires thymectomy (153). Over the years, additional strategies have been explored to prolong graft survival mediated by anti-CD40L antibody. Most approaches make use of blocking antibodies to other costimulatory molecules, such as CD28 and OX40, or agonistic antibodies for coinhibitory molecules, such as CTLA-4 (154). More recently, the roles of newer members in the costimulation family have been targeted in combination with anti-CD40L, such as PD1/PD-L1 and the ICOS/B7RP-1 (155–157). In addition, antibodies interfering with adhesion directly or indirectly, such as lymphocyte function-associated antigen-1 (LFA-1) and/or CD45RB have shown to be beneficial (158–163). Additionally, promising results come from studies targeting IL-2, IL-7, and IL-15 in combination with CD40L antibody (164–166) because it was shown that signaling through the common  $\gamma$ -chain was the reason why diabetic mice were resistant to CD40/CD28-induced tolerance (167).

### Additive suppression by blocking other costimulatory pathways

The clonal deletion of alloreactive T cells can be accelerated in the absence of CD80/CD86 expression and in the absence of CD40L costimulation (147). Blocking of either pathway will lead to prolonged graft survival in some models. Anti-CD28 or anti-CD80/86 antibody is based on blocking costimulation. Although both approaches target the same pathway, the anti-CD28 antibody is mainly based on passive apoptosis of T cells, whereas the anti-CD80/86 antibody is based on actively inducing cell death. Therefore, CD40 resembles anti-CD28 blockade with combination therapies, showing variable results on graft survival (127, 150, 168–170). In addition to blocking costimulation, the engagement of CD80/CD86 by CTLA-4-Ig, has been shown to upregulate the expression of indole amine 2,3-dioxygenase (IDO), a process believed to normally occur by the interaction of regulatory T cells and DCs (171). IDO degrades tryptophan leading to local removal of this essential aa needed for T-cell proliferation (172). However, regulatory T cells may also mediate suppression by eliciting IDO from DCs (171).

Using double knockouts for CD28 and CD40L revealed that these mice could still reject allografts and it was shown that the OX40/OX40L pathway was critical in this process. The 4-1BB/4-1BBL, ICOS/B7RP-1 or CD27/CD70 pathways were not involved in facilitating graft rejection in the double-knockout mice (173). Later, it was shown that OX40 played an important role in rejection mediated by memory T cells (157). OX40 signaling, in addition to activating effector T cells also turns off the suppressive function of regulatory T cells (174). It must be noted that multiple publications have shown that blocking the OX40/OX40L pathway in the absence of CD40L leads to long-lasting pancreatic islet graft survival (175). The effect of OX40L blockade is mainly because of impairment of rejection, which is mediated by CD4<sup>+</sup> T cells and to a lesser extent to CD8<sup>+</sup> T cell (173, 176).

In conclusion, the blocking of CD40L leads to prolonged graft survival and seems to be synergistic when combined with most of the other members of both the TNF-family and B7-family of costimulatory molecules. Although it seems that CD40 and CD28 are the most prominent members in the regulation of peripheral tolerance, there are additive, but redundant roles of the other family members impacting specific subset of T cells involved in maintaining tolerance.

### CD40-based immunotherapy in cancer

CD40/CD40L is critical for the development of protective anti-tumor immunity and CD40 can be a cancer-associated target in antibody-based therapies. In fact, CD40 was first

discovered as an antigen expressed in bladder carcinoma and paralleled its identification as a receptor on B cells (13, 177–181).

### CD40 expression in tumors

The broad range of expression of CD40 on normal healthy cells translates to its extensive expression on a variety of tumors. Reports have shown that CD40 is widely expressed on both murine and human melanoma, prostate, and lung cancers (182, 183), as well as in carcinomas of the nasopharynx, bladder, cervix, and ovary (184–187). CD40 expression has been reported on both non-Hodgkin's lymphomas (NHLs) and Hodgkin's lymphomas and on other hematologic malignancies, such as lymphocytic leukemia, lymphoma, multiple myeloma, and acute myeloid leukemia (188–191).

### Direct effect of CD40/CD40L interaction on tumor cells

As in normal B cells, CD40 ligation in certain B-cell malignancies causes an increase in the expression of anti-apoptotic factors such as Bcl-XL, TNF $\alpha$ -induced protein 3 (A20), Bcl/B-cell lymphoma 2 related protein A1a (Bfl-1), survivin, and cFLIP. These factors protect the cell from apoptosis induced by apoptotic agent, such as, serum withdrawal, IgM, anti-Fas, TNF-related apoptosis-induced ligand (TRAIL) or DNA-damaging agents (109, 192–195). It has been suggested that low-level constitutive engagement of CD40 may facilitate malignant cell growth. Studies with non-Hodgkin's lymphoma, Burkitt's lymphoma, and chronic lymphocytic leukemia cells have shown that these cells express low levels of CD40L, and through an autocrine pathway sustain cell proliferation. In addition, the low-levels of CD40L expression protect these cells from apoptosis (196–198). Recently, human CD40-positive breast tumor biopsies were shown to co-express CD40L, and that co-expression confers oncogenic effects *in vitro* (177). Furthermore, the co-expression of CD40 and its ligand, in immortalized human epithelial cells induce an increase in their proliferation, motility, and invasion (177). These results suggest that neoplastic growth utilizes the CD40/CD40L pathway independent of the immune system to sustain proliferative capacity and survival. Furthermore, by expression of the receptor/ligand pair, tumors are able to manipulate both T-cell and APC compartments most likely contributing to the establishment of the immunosuppressive tumor microenvironment.

Conversely, transient activation of CD40 on carcinomas and some B-cell malignancies results in direct anti-proliferative effects and apoptosis. Both *in vitro* and *in vivo* treatment of Burkitt's lymphoma, primary high-grade B-cell lymphoma, and multiple myeloma cells with CD40L results in a reduction in the proliferation of these tumor cells (189, 199). Under CD40 stimulation, carcinoma cells can be induced to undergo apoptosis by the increased expression of Bcl2-associated X protein and the upregulation of membrane-bound cytotoxic ligands of the TNF family, such as FasL, TNF, and TRAIL (200, 201). Cell death induction seen by CD40L occurs even when protein synthesis is blocked, suggesting that the production of anti-apoptotic factors may override the cell death induction signal provided by CD40L (202, 203). CD40L triggering of bladder and ovarian carcinomas *in vitro* results in growth inhibition and enhanced susceptibility to apoptosis, induced by anti-neoplastic drugs, TNF $\alpha$ , Fas, and ceramide (203). These observations were confirmed in an *in vivo* system, where CD40L alone or in combination with chemotherapy could significantly inhibit the growth of transplantable breast or ovarian tumors and confer overall survival (204, 205). Hence, CD40 activation can have a direct anti-tumor effect on several tumors, even in the absence of any additional immune responses and cells. The direct cytotoxic effect of CD40L on CD40-expressing tumors has been studied using immuno-compromised mice, where it was observed that inhibition of breast carcinoma and B-cell lymphoma growth (199, 200) can be observed in the absence of an intact immune system.

In summary, CD40 activation can induce the function of various downstream signaling pathways including both, pro-apoptotic and anti-apoptotic proteins. It is clear that the function of direct CD40 stimulation on malignancies is dependent on the state of differentiation of the stimulated cells and the type of malignancy, as reviewed extensively elsewhere (206).

### **CD40/CD40L is central to the development of protective anti-tumor immunity**

CD40 is a potent stimulator of the immune system and, as such, CD40/CD40L interaction on immune cells has been extensively studied for its involvement in the development of protective anti-tumor immunity. The first report implicating CD40/CD40L interaction as a critical factor in the generation of protective immunity by means of tumor vaccines came from Mackey *et al.* (207). It was shown that anti-CD40L monoclonal antibody treatment inhibited the generation of protective immune responses and prevented the therapeutic value of potent tumor vaccines. These results were confirmed using CD40-deficient mice. These mice were unable to generate a protective anti-tumor immune response following a protective vaccination regime (207).

Cognizant of the fact that CD40L was critical for inducing protective tumor immunity, it was anticipated that agonistic anti-CD40 antibodies would be powerful adjuvants for induce tumor immunity. Indeed, early studies showed that CD40 agonistic antibody generated CTL responses that were able to eradicate tumor in a lymphoma system. It was also seen that CD40 ligation could overcome peptide-induced peripheral CTL tolerance and increase anti-tumor vaccine efficacy (208–210). However, use of CD40 as a solitary agonist to induce immunity came into question.

Several reports have confirmed that CD40 stimulation can enhance anti-tumor immune responses by means of DC maturation (211). Activation of DCs with agonist of CD40 results in their increased survival, secretion of IL-1, IL-6, IL-8, IL-12, TNF- $\alpha$ , and macrophage inflammatory protein-1 $\alpha$ . Additionally, CD40 activation induces the upregulation of costimulatory molecules such as MHC class II, LFA-3, CD80, and CD86 and promotes antigen presentation, priming and cross-priming of Th and CTL, respectively (206). However, the use of agonist anti-CD40 antibody alone accelerates the deletion of tumor-specific CTL in the absence of antigen vaccination in a mouse-melanoma model system (212). While immunotherapeutic regimes utilizing CD40-dependent IFN-responses lead to successful early anti-tumor efficacy, the treatment with CD40 agonist alone impairs the development of tumor-specific T cells (180). It is clear that robust development of adaptive immunity relies on the interplay of both innate and adaptive immunity, and therefore the contribution of Toll-like receptor (TLR) agonists to CD40-dependent stimulation of adaptive immunity was evaluated. Recently, our group showed that the combinatorial use of CD40 and TLR agonists induces profound frequencies of anti-tumor-specific T cells and provides therapeutic efficacy in a number of tumor models. An increase in the frequencies of tumor-reactive CD8<sup>+</sup> T cells that efficiently infiltrate the tumor-burdened organ resulted in the generation of potent tumor-specific memory CD8<sup>+</sup> T cells. These results suggest that future CD40-dependent anti-tumor approaches should encompass activators of both innate and adaptive immune systems (213).

Studies seeking to utilize CD40 ligation for tumor immunotherapy have been approached using gene delivery of the CD40L gene to DCs and tumor cells. Expression of CD40L in a small proportion of tumor cells was enough to generate a long-lasting systemic anti-tumor immune response in mice that was shown to be dependent in CTLs (214). Further, it was demonstrated that CD40 ligation can bypass the absolute requirement of CD4<sup>+</sup> T cells during immunization with melanoma antigen gene-modified DCs (215). Other gene therapies have been successful in murine tumor models, e.g. *ex vivo* virus delivery to either

DC or tumors by use of recombinant adenovirus expressing CD40L. This approach has proven effective in syngeneic tumor models of colorectal carcinoma, lung carcinoma and melanoma (216, 217). It is likely that CD40 monotherapy is effective in these models because the adenoviral delivery system provides the other necessary TLR signal to drive the development of therapeutic immunity.

### **The experience of targeting CD40 in human cancer clinical trials**

To date, three main rationales have been used in the development of clinical phase I studies which target the CD40 pathway. First, that CD40 can act as a tumor-associated antigen to which a cytotoxic antibody can be targeted. Second, CD40 engagement by an antibody can transduce a pro-apoptotic signal to a tumor cell to facilitate its death. Third, an agonistic anti-CD40 antibody can enhance immunity to the tumor and facilitate the development of therapeutic immunity.

A summary of some of the CD40-directed clinical studies can be found in Table 1 where we list the three main approaches to CD40 targeting. These three are CD40L gene therapy, recombinant protein therapy, and anti-CD40 therapy (179, 181, 218–226). Most of the reported therapies are relatively well tolerated, although each treatment exhibits some adverse reactions.

The use of a human trimeric recombinant CD40L in patients with solid tumors was development based on the pro-apoptotic activities of this molecule on human tumors in recombination gene knockout mice (227). Patients with advanced solid tumors or intermediate- or high-grade NHL received CD40L subcutaneously daily for 5 days in a phase I dose-escalation study. Doses continued until disease progression was noted. CD40L induce liver toxicity noted by the increased levels of liver transaminases in the serum. Six percent of patients had a partial response with one patient having a complete response. Since the time of the study in 2001, no further studies have been reported (219).

Humanized or fully human monoclonal IgG antibodies have since been developed, such as, CP-870,893 (Pfizer Inc., New York, NY, USA), SGN-40 (Seattle Genetics Inc., Bothell, WA, USA), and HCD 122 (Novartis/XOMA Basel, Switzerland). For the Pfizer studies, patients in this phase I trial with advanced solid tumors received single doses of the CD40 agonistic antibody, CP-870,893 intravenously. Dose-limiting toxicity was observed with the most common adverse event being cytokine release syndrome including chills, rigors, and fever. 14% of all patients and 27% of melanoma patients had objective partial responses at restaging. CP-870,893 was well tolerated and was shown to be biologically active, and was associated with anti-tumor activity. The authors concluded that further studies were warranted. While the presumed mechanism of active are still unresolved, the intent is that CP-870,893 will induce tumor immunity.

Dacetuzumab (SGN-40) is a weak agonist and is currently in multiple phase II trials in multiple myeloma and diffuse large-cell lymphoma. Seattle Genetics reported in December of 2008 that data from a phase II trial demonstrated objective responses at well-tolerated doses in heavily pretreated patients with diffuse large B-cell lymphoma. SGN-40 is a weak agonist and is believed to trigger apoptosis.

A phase I trial with HCD122, a CD40 antagonist, was well tolerated in patients with relapsed and refractory multiple myeloma. The majority of adverse events were mild to moderate in severity. HCD122 monotherapy achieved a partial response in one out of the six patients treated. HCD122 is believed to facilitate tumor apoptosis by depriving B-cell tumors of growth promotion via CD40 and mediating lysis by antibody-dependent cellular cytotoxicity (228). The most likely direction toward which CD40-directed tumor therapy

should be moving is in combination with alternative or more established therapies, such as radiation, cancer vaccine, chemotherapy, anti-CTLA-4 antibody blocking, TLR agonists, and cytokines (205, 209, 210, 213, 229, 230).

In conclusion, the overall effect of CD40 therapy in the activation of the immune system and direct tumor cytotoxicity is most likely to act synergistically in the purpose of establishing an anti-tumor effect. Additionally, it is currently generally agreed upon that optimal DC activation depends on synergistic triggering of several ‘danger’ receptors by the administration of exogenous TLR ligands and CD40 agonists to elicit maximum anti-tumor T-cell response or therapeutic effect. Taking lessons from the last few decades of anti-cancer drug development, it is currently pretty well accepted that the cure for or treatment of tumors will have to be a combinatorial approach that acts both on the tumor itself, as well as on the level of immune response induction.

## Conclusion

The wide-range expression of CD40 in normal as well as tumor cells shows the pivotal role that CD40 is playing in diverse cellular and immune processes. The understanding of CD40 signaling in a humoral and cellular context will address to improve therapies against pathogens and tumors. In contrast, the blocking of CD40/CD40L engagement will drive to generation of tolerance during transplant or in autoimmunity diseases.

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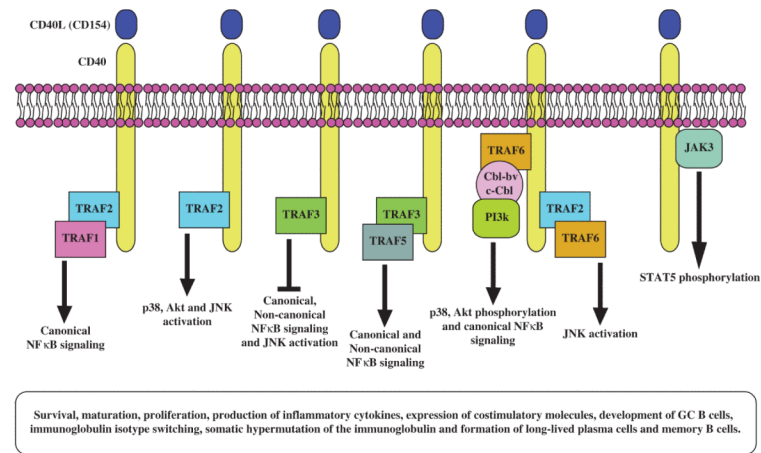
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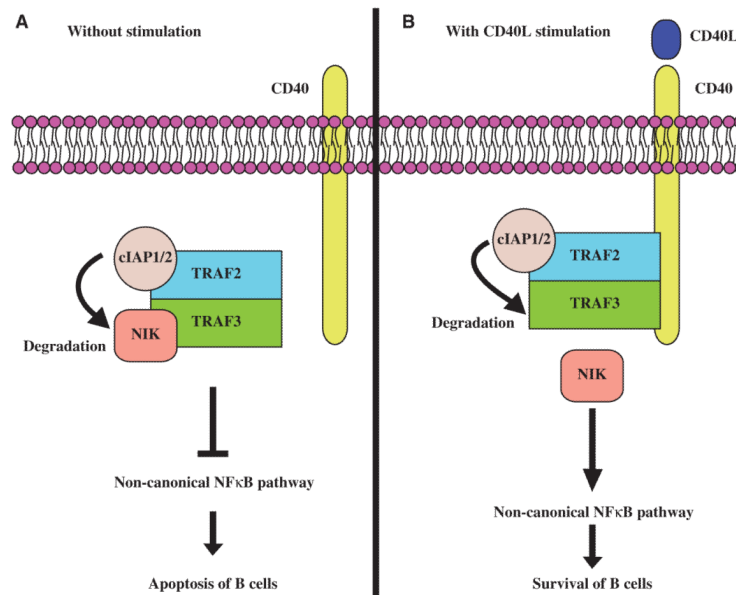


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**Fig. 1. CD40 signaling dependent and independent of tumor necrosis factor receptor-associated factor (TRAF) proteins**

After CD40 activation, TRAFs 1, 2, 3, 5, and 6 are recruited to CD40 tail, driving different signaling pathways. Furthermore, Janus family kinase 3 can bind to the proximal cytoplasmic membrane of CD40. The signaling dependent or independent of TRAF protein regulates different cellular and immune processes.



**Fig. 2. Role of tumor necrosis factor receptor-associated factors (TRAFs) 2 and 3 in the inhibition of non-canonical nuclear factor  $\kappa$ B (NF  $\kappa$ B) pathway**

Under non-stimulation, TRAF2 and TRAF3 form a complex with cellular inhibitor of apoptosis 1 and 2 (cIAP1 and cIAP2) and NF  $\kappa$ B-inducing kinase (NIK). cIAP1/2 degrades NIK, ablating the non-canonical NF  $\kappa$ B pathway (39, 40). After CD40/CD40L engagement, the complex is destabilized, permitting the release of NIK from the complex inducing the non-canonical NF  $\kappa$ B signaling. Furthermore, it induced the recruitment of TRAF2 and TRAF3 to CD40 tail and the degradation of TRAF3 by cIAP1/2 proteins.

Table 1

Human clinical trial therapy in cancer patients

Tumor	Treatment	Clinical observations	Mechanism	References
CD40L gene therapy B-CLL ( <i>n</i> = 11)	CD40L-transfected leukemia cells, I.V. transfer	No severe adverse reaction Flu-like symptoms Reduced leukemic cell count (>50% at 2–4 weeks postinfusion and lymph node size (>90% reduction))	T-cell sensitization Agonist	Wierda <i>et al.</i> (221) Kipps <i>et al.</i> (222)
CD40L-expressing leukemia cells with IL-2-expressing fibroblasts ( <i>n</i> = 10)	CD40L and IL-2 transduced leukemic blasts and skin fibroblasts, S.C. transfer	No severe adverse reaction 90% 5-year survival rate	Cytotoxic effector-cell activity Generation of IgG Ab Agonist	Rousseau <i>et al.</i> (226)
Recombinant protein therapy Solid carcinomas and NHL ( <i>n</i> = 32)	Recombinant CD40L, S.C. injections, daily ×5	Some adverse reaction Injection site reaction Increased AST/ALT 38% Responders with stable disease 2 Partial responses	Undetermined Agonist	Vonderheide <i>et al.</i> (219) Younes <i>et al.</i> (220)
Anti-CD40 monoclonal antibody therapy Advanced solid tumors ( <i>n</i> = 29)	CP-870,893 (human IgG2, Pfizer, New York, NY, USA), I.V. infusion	Some adverse reaction	Transient depletion of CD19 <sup>+</sup> B cells in blood	Vonderheide <i>et al.</i> (219)
NHL ( <i>n</i> = 29)	SGN-40 (humanized IgG1, Seattle Genetics, Bothell, WA, USA), I.V. infusion	Cytokine release syndrome (chills, rigors, fever) 14% Partial responses of all patients and 27% in melanoma patients	Agonist	Forero-Torres <i>et al.</i> (218)
Multiple myeloma ( <i>n</i> = 23)		Some adverse reactions Cytokine release syndrome 14% Partial response and 3.5% complete response	Weak agonist	
		Some adverse reaction Cytokine release syndrome 17% of patients had decreases in M-protein	CD19 <sup>+</sup> B cell decrease Weak agonist	Hussein <i>et al.</i> (225)
CLL and multiple myeloma ( <i>n</i> = 24)	HCD 122 (human IgG1, Novartis/XOMA, Basel, Switzerland)	Some adverse reactions	Blocks CD40L-induced cell survival	Byrd <i>et al.</i> (181)
		Transient infusion reactions 8% Partial response	Mediates ADCC Antagonist	Bensinger <i>et al.</i> (179)

B-CLL, B and chronic lymphocytic leukemias; I.V., intravenous; S.C., subcutaneous; AST, aspartate aminotransferase; ALT, alanine aminotransferase; NHL, non-Hodgkin's lymphoma, Ig G, immunoglobulin G; IL-2, interleukin-2.